User's Manual and Instructions

Product: RapidSeq Small RNA Sample Prep Kit

Catalog Number: KS071012, KS071012-I, KS071012-II, KS071012-III, and KS071012-IV

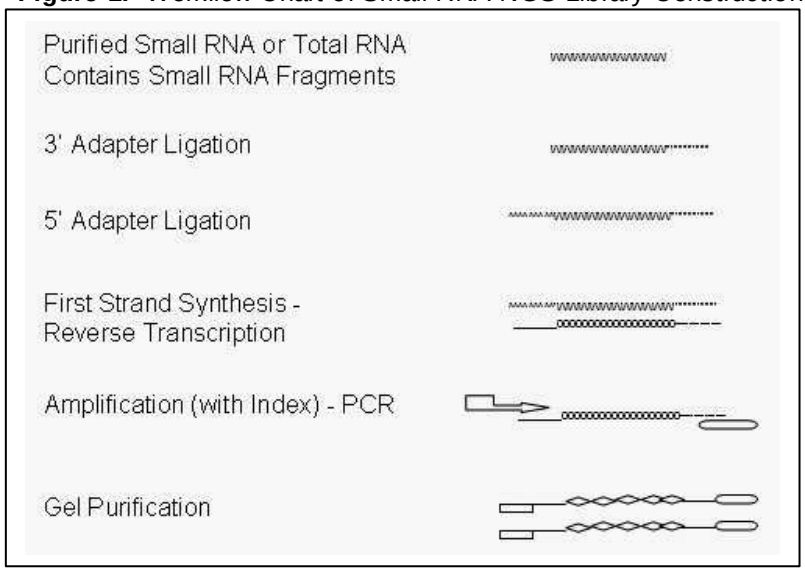
Introduction

Small RNA includes microRNA (miRNA), ncRNA, siRNA, snoRNA, piRNA, rasiRNA, and many more. It is a large family of regulatory molecules in organisms and plays an important role in development and disease. Next Generation Sequencing (NGS) is a powerful tool to identify and quantitatively analyze the entire population of small RNAs.

miRNAs are endogenous regulators of gene expression that are encoded in the genomes of animals, plants and viruses. Mature miRNAs are 18-24 nt, single-stranded molecules that become incorporated into the RNA-induced silencing complex (RISC). RISC mediates down-regulation of gene expression through translational inhibition, transcript cleavage, or both.

This manual aims to prepare NGS libraries for subsequent cluster generation, using purified small RNA or total RNA which contains small RNA fragments as input. The protocol includes steps for adapters ligation, reverse transcription, PCR amplification, and size selection by gel purification to generate a library product compatible with illumina NGS platform (Figure 1). The method in this kit ligates adapters directionally to miRNAs based on their nature structure with a 5'- phosphate and a 3' - hydroxyl group.

Figure 1. Workflow Chart of Small RNA NGS Library Construction



BioChain also provides other tools and services to researchers interested in studying small RNA. Please contact BioChain Technical Support for further details.

Features

- Simple workflow - most components are supplied as ready-to-use super mixtures which reduces setup time and liquid handling steps (Table 1)
- Great performance - comparable yield with benchmark's fresh made mixtures
- Wide dynamic range - total RNA input could be down to 100 ng

Table 1. Savings in manual time and effort with BioChain method

Protocol	Hands on time	Total process time
3' Adapter Ligation	<10 min	1 hr 15 min
5' Adapter Ligation	5 min	1 hr
First Strand Synthesis	5 min	1 hr
PCR	5 min	40 min
Size selection	10 min	1 hr
Purification	5 min	2 hr
Total workflow time	**<40 min**	**<7 hr**

Applications

- Small RNA detection and quantification
- Small RNA discovery
- MiRNA expression profiling
- MiRNA related functional assessment and validation

Description

Components in this kit are prepared with pure chemicals to construct NGS libraries compatible with Illumina's sequencing platform for subsequent cluster generation, using purified small RNA or total RNA contains small RNA fragments as input. 4 sets of the kit with different 4 sets of 12 aligners, respectively are available.

Quality Control

At least one kit of each lot has been tested for small RNA NGS library construction using BioChain's Adult Normal Lung Tissue Total RNA (Cat# R1234152-50) and Illumina's NGS instrument. Good coverage and low adapter dimer are observed. All known miRNAs are captured.

Components

One kit has 3 boxes listed in below, only one aligner box is included in one kit (see table 2-4 below). Reagents are sufficient for 12 assays.

Table 2. Contents List of RapidSeq Small RNA Sample Prep Kit (Box 1 of 3)

Cap Color	Item	Amount in kit	Part No.
Green	Tail Oligo	14 µl	KS071012-1
	Tail MasterMix	53 µl	KS071012-2
	Ligation Enhancer	14µl	KS071012-3
Red	Cap Oligo	14 µl	KS071012-4
	Cap MasterMix	26.5 µl	KS071012-5
Yellow	RT Oligo	14 µl	KS071012-6
	RT MasterMix	75 µl	KS071012-7
Blue	Universal Primer	28 µl	KS071012-8
	PCR MasterMix	340 µl	KS071012-9
Nature	Nuclease-free Water	500 µl	KS071012-10
Amber	Gel Cutting Indicator	26 µl	L5022100-DS

Table 3. Contents List of RapidSeq Small RNA Sample Prep Kit (Box 2 of 3)

Item	Amount in kit	Part No.
Gel Cutter	12	KS071012-11
Gel Breaker	12	KS071012-12
Gel Filter	12	KS071012-13
DNA Storage Solution	1500 µl x 2	LB3401010

Table 4. Contents List of RapidSeq Small RNA Sample Prep Kit (Box 3 of 3)

KS071012-I

Item	Amount in kit (µl)	Part No.	Sequence
Aligner 1	10	KS072012-1	ATCACG
Aligner 2	10	KS072012-2	CGATGT
Aligner 3	10	KS072012-3	TTAGGC
Aligner 4	10	KS072012-4	TGACCA
Aligner 5	10	KS072012-5	ACAGTG
Aligner 6	10	KS072012-6	GCCAAT
Aligner 7	10	KS072012-7	CAGATC
Aligner 8	10	KS072012-8	ACTTGA
Aligner 9	10	KS072012-9	GATCAG
Aligner 10	10	KS072012-10	TAGCTT
Aligner 11	10	KS072012-11	GGCTAC
Aligner 12	10	KS072012-12	CTTGTA

KS071012-II

Item	Amount in kit (µl)	Part No.	Sequence
Aligner 13	10	KS072012-13	AGTCAA
Aligner 14	10	KS072012-14	AGTTCC
Aligner 15	10	KS072012-15	ATGTCA
Aligner 16	10	KS072012-16	CCGTCC
Aligner 17	10	KS072012-17	GTAGAG
Aligner 18	10	KS072012-18	GTCCGC
Aligner 19	10	KS072012-19	GTGAAA
Aligner 20	10	KS072012-20	GTGGCC
Aligner 21	10	KS072012-21	GTTTCG
Aligner 22	10	KS072012-22	CGTACG
Aligner 23	10	KS072012-23	GAGTGG
Aligner 24	10	KS072012-24	GGTAGC

KS071012-III

Item	Amount in kit (µl)	Part No.	Sequence
Aligner 25	10	KS072012-25	ACTGAT
Aligner 26	10	KS072012-26	ATGAGC
Aligner 27	10	KS072012-27	ATTCCT
Aligner 28	10	KS072012-28	CAAAAG
Aligner 29	10	KS072012-29	CAACTA
Aligner 30	10	KS072012-30	CACCGG
Aligner 31	10	KS072012-31	CACGAT
Aligner 32	10	KS072012-32	CACTCA
Aligner 33	10	KS072012-33	CAGGCG
Aligner 34	10	KS072012-34	CATGGC
Aligner 35	10	KS072012-35	CATTTT
Aligner 36	10	KS072012-36	CCAACA

KS071012-IV

Item	Amount in kit (µl)	Part No.	Sequence
Aligner 37	10	KS072012-37	CGGAAT
Aligner 38	10	KS072012-38	CTAGCT
Aligner 39	10	KS072012-39	CTATAC
Aligner 40	10	KS072012-40	CTCAGA
Aligner 41	10	KS072012-41	GACGAC
Aligner 42	10	KS072012-42	TAATCG
Aligner 43	10	KS072012-43	TACAGC
Aligner 44	10	KS072012-44	TATAAT
Aligner 45	10	KS072012-45	TCATTC
Aligner 46	10	KS072012-46	TCCCGA
Aligner 47	10	KS072012-47	TCGAAG
Aligner 48	10	KS072012-48	TCGGCA

Storage and Stability

Upon receipt, store all reagents appropriately. Avoid repeated freeze/thaw cycles. This kit is stable for half a year after shipping date.

Protocol

Consumables Preparation

The kit has all key reagents to run experiment but not common consumables and instruments. Please make sure all needs are available before starting this protocol (Table 5).

Table 5. List of Consumables

Consumable	Supplier
0.2 ml, 1.5 ml, and 2 ml clean, nuclease - free microcentrifuge tubes	General lab supplier
200 µl, clean, nuclease - free PCR tubes	General lab supplier
5X Novex Hi-Density TBE Sample Buffer	Invitrogen, LC6678
5X Novex TBE Buffer	Invitrogen, LC6675
6% Novex TBE PAGE Gel, 1.0 mm, 10 well	Invitrogen, EC6265BOX
DNA 1000 chip	Agilent, 5067 - 1504
Ultra Pure Ethidium Bromide	General lab supplier
High Sensitivity DNA chip	Agilent, 5067 - 4626

Cautions

1. This product is for ***Research Use Only***.
2. Close adherence to the protocol will assure optimal performance and reproducibility.
3. Set up reactions in sterile, nuclease - free tubes on ice.
4. Prepare 10% extra mixture when running multiple samples.
5. Care should be taken to ensure nuclease - free processing.
6. Due to the analytical sensitivity of this test, extreme care should be taken to avoid the contamination of reagents.
7. The assay kit should be used as a system. Do not substitute other manufacturer's reagents. Dilution, reducing reaction volumes, or other deviation in this protocol may affect the performance of this testing kit.
8. Do not mix or combine reagents from kits with different lot numbers.
9. Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.

RNA Input

1. This protocol has been optimized using 1 µg of high quality human lung total RNA as input. Use of degraded RNA can result in low yield.
2. Purified 1~10 ng small RNA or miRNA from total RNA can also be used as starting material. Small RNA populations can vary significantly between different tissue types and species. Use of RNA from other species, tissues, or qualities may require further optimization.
3. BioChain recommends using Adult Lung Tissue Total RNA (catalog #R1234152-50) as a positive control sample for this protocol. This product is certified to contain the small RNA fraction.

Pooling

Each RapidSeq Small RNA Sample Prep Kit can be used to construct libraries that are compatible with illumina multiplexing, with up to 12 samples combined into a single lane. While processing samples in parallel, incorporate the index at the amplification step following reverse transcription. Samples could be pooled immediately prior to gel purification.

Library Preparation

Pre-heat the thermal cycler to 70°C and pre-heat another thermal cycler to 28°C if available.

1. Briefly centrifuge the thawed reagents at 600 xg for 5 seconds, then place them on ice.
2. Prepare RNA sample for total volume at **5** µl (use Nuclease-free Water as dilution if necessary) in a sterile, nuclease-free 200 µl PCR tube on ice.
3. Add **1** µl Tail Oligo into RNA tube. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly.
4. Incubate the tube at 70°C for 2 minutes and then immediately place the tube on ice.
5. Add **4** µl of Tail MasterMix to the reaction tube. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.
6. Incubate the tube at 28°C for 1 hour.
7. Directly add **1**ul Ligation Enhancer into each reaction tube remaining on the thermal cycler, gently pipette the entire volume up and down 6–8 times to mix thoroughly, continue incubate the tube at 28°C for 15 minutes and then place the tube on ice.
8. Aliquot **1** µl Cap Oligo into a separate, nuclease-free 200 µl PCR tube, incubate at 70°C for 2 minutes and then immediately place the tube on ice.
9. Add **2** µl of Cap MasterMix to Cap Oligo tube for each reaction. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.
10. Transfer these **3** µl of the Cap mixture to the Tail reaction tube. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.
11. Incubate at 28°C for 1 hour and then place the tube on ice.
12. Aliquot **6** µl of the whole reaction into a separate, sterile, nuclease-free, 200 µl PCR tube. (Left could be stored at -80°C.)
13. Add **1** µl RT Oligo. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly.
14. Incubate at 70°C for 2 minutes and then immediately place the tube on ice.
15. Pre-heat the thermal cycler to 50°C.
16. Add **5.5** µl of RT MasterMix. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly.
17. Incubate at 50°C for 1 hour and then place the tube on ice.
18. In a separate, sterile, nuclease-free, 200 µl PCR tube, set up PCR mixture as below.

Mixture	**µl**
PCR MasterMix	25
Universal Primer	2
Aligner*	2
Nuclease-free Water	8.5
Total	**37.5**

* For each reaction, only one of the 48 Aligners is used during this step.

Gently pipette the entire volume up and down 6–8 times to mix thoroughly, centrifuge briefly, then place the tube on ice.

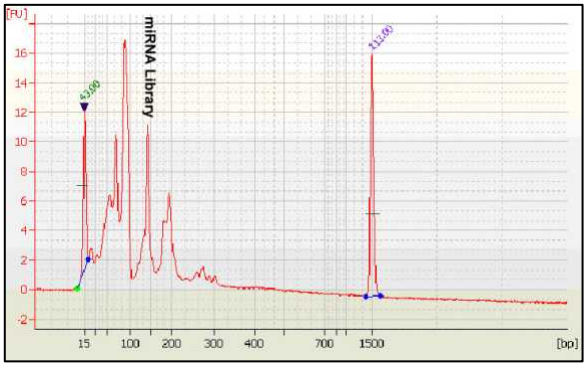
19. Transfer this **37.5** µl mixture to the RT reaction tube. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly and place the tube on ice.
20. Amplify the tube in the thermal cycler using the following PCR cycling conditions:

1): 98°C for 30 seconds; 2): 11 cycles of: 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 15 seconds; 72°C for 10 minutes; 3): Hold at 4°C.

Amplification products may vary based on RNA input amount, tissue type, and species. This process was optimized using 1 µg of Adult Lung Tissue Total RNA. The number of PCR cycles can be adjusted to a maximum of 15 cycles if no clear bands in the gel image.

21. Run sample on a DNA1000 chip according to the manufacturer's instructions. The following figure 2 shows typical results from Adult Normal Lung Tissue Total RNA (Cat# R1234152-50).

Figure 2. Adult Normal Lung Tissue Total RNA Sample Trace of Amplicons on DNA1000 Chip

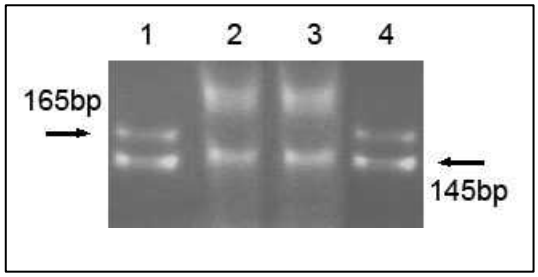


Size Selection by Gel Purification

1. Assemble the gel electrophoresis apparatus per the manufacturer's instructions with appropriate amount of 1X TBE Running Buffer.
2. Mix 2 µl of Gel Cutting Indicator with 2 µl of DNA Loading Buffer (5X Novex Hi-Density TBE Sample Buffer or equivalent).
3. Mix amplified cDNA library with appropriate amount of DNA Loading Buffer.
4. Load 2 µl per lane of Gel Cutting Indicator in outer side of sample wells.
5. Load maximum 30 µl cDNA library each well in between two Indicator wells.
6. Run the gel for 60 minutes at 145 V or until the blue front dye exits the gel.
7. Remove the gel from the apparatus and open the cassette according to the manufacturer's instructions.
8. Stain the gel with Ethidium Bromide (0.5 µg/ml in water) in a clean container for 2 - 3 minutes.
9. View the gel on a Dark Reader transilluminator or a UV transilluminator.

The following figure 3 shows gel analysis of an Adult Normal Lung Tissue small RNA library.

Figure 3. Small RNA Library from an Adult Normal Lung Tissue Total RNA Sample

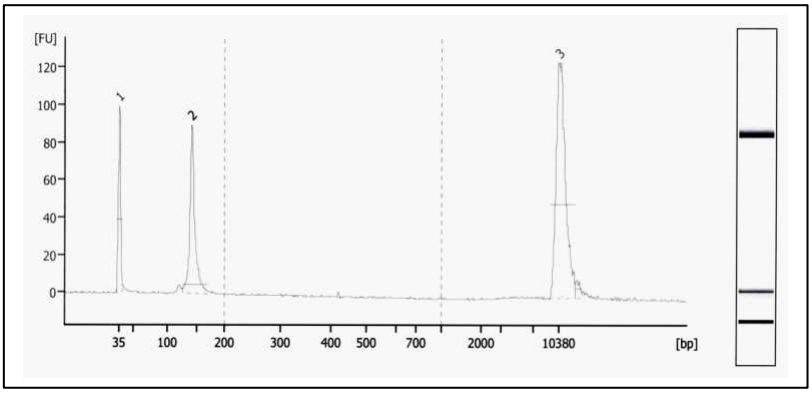


* Lane 1 and 2: Gel Cutting Indicator for miRNA NGS library size selection;

Lane 3 and 4: Amplicons of an Adult Normal Lung Tissue Total RNA Samples

10. Place the gel breaker tube into a sterile, round - bottom, nuclease - free, 2 ml microcentrifuge tube.
11. Using a Gel Cutter, cut out miRNA NGS library band between two Cutting Indicators and excise the gel fragment.
12. Place the band of interest into the 0.5 ml Gel Breaker tube.
13. Centrifuge the stacked tubes to 20,000 g in a microcentrifuge for 2 minutes at room temperature. Ensure that the gel has all moved through the holes into the bottom tube.
14. Remove Gel Breaker tube, add 200 µl of DNA Storage Solution to the gel debris in the 2 ml tube.
15. Elute the DNA by shaking the tube around 1300 rpm at room temperature for at least 2 hours or overnight if desired.
16. Transfer the eluate and the gel debris to the top of a 5 µm filter.
17. Centrifuge the filter for 10 seconds to 600 g and then discard the filter.
18. Check the size, purity and concentration of the library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip (Figure 4).

Figure 4. High Sensitivity DNA Chip Trace of the Final Library from an Adult Normal Lung Tissue Total RNA Sample



* Peak 1: Lower Marker; Peak 2: miRNA NGS Library; Peak 3: Upper Marker

Related Products

MagSeq mRNA Purification Kit (Cat# K2012008)
MicroRNA Isolation Kit (Cat# KS 341025)
Broad Range Total RNA Isolation Kit (Cat# K1341050)
BioChain Total RNA (containing miRNAs)

References

1. Cullum R, *et al. Respirology* 2011. 16:210-222.
2. Shalgi R, *et al. Aging* 2009. 1:762-770.
3. Ach, R., *et al. BMC Biotechnology* 2008. 8:69.